SYNOVIUM-DERIVED STROMAL CELL-INDUCED OSTEOCLASTOGENESIS: A POTENTIAL OSTEOARTHRITIS TRIGGER.

Manuela Dicarlo, PhD[1]*, Gabriella Teti, PhD[2]*, Giorgia Cerqueni, BS[3], Iolanda Iezzi,MD [3], Antonio Gigante,MD[3], Mirella Falconi,MD[2], Monica Mattioli-Belmonte,PhD[3#]

*Both Authors Equally contributed to the Research

[1] Laboratory of Experimental Immunopathology, IRRCS "S de Bellis", Castellana Grotte, 70013, Bari, Italy

[2] Department of Biomedical and Neuromotor Sciences-DBNS, Università di Bologna, Via Irnerio 48, 40126 Bologna, Italy.

[3] Department of Clinical and Molecular Sciences (DISCLIMO), Università Politecnica delle Marche, 60126, Ancona, Italy [#]Correspondence to: m.mattioli@univpm.it

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Statement of author contributions:

Prof. Mattioli-Belmonte planned and oversaw the whole research; Prof. Gigante furnished tissue samples and clinical suggestion; Dr. Dicarlo executed cell cultures; Dr. Cerqueni performed immunohistochemical analysis; Prof. Teti was responsible for ultrastructural investigation; Dr. Iezzi was responsible for qRT-PCR analysis; Prof. Falconi oversaw morphological analyses. All authors equally and competently contributed to the draft.

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Abstract

Purpose: To shed light on the idea that mesenchymal stem/stromal cells recruited in synovium (Synovium-Derived Stromal Cells-SDSCs) could be involved in Osteoarthritis (OA) pathophysiology. Attention was also paid to a further stromal cell type with a peculiar ultrastructure called telocytes (TCs), whose role is far to be clarified. **Methods:** In the present *in vitro* study we compared SDSCs isolated from healthy and OA subjects in terms of phenotype, morphology and differentiation potential as well as in their capability to activate normal Peripheral Blood Mononuclear Cells (PBMCs). Histological, immunohistochemical and ultrastructural analyses were integrated by qRT-PCR and functional resorbing assays. **Results:** Our data demonstrated that both SDSC populations stimulated the formation of osteoclasts from PBMCs: the osteoclast-like cells generated by healthy-SDSCs via trans-well co-cultures were inactive, whilst OA-derived SDSCs have a much greater effectiveness. Moreover, the presence of TCs was more evident in cultures obtained from OA subjects and suggests a possible involvement of these cells in OA. **Conclusions:** Osteoclastogenic differentiation capability of PBMCs from OA subjects, also induced by B synoviocytes has been already documented. Here we hypothesized that SDSCs, generally considered for their regenerative potential in cartilage lesions, have also a role in the onset/maintenance of OA. **Clinical Relevance:** Our observations may represent an interesting opportunity for the development of a holistic approach for OA treatment, that considers the multifaceted capability of MSCs in relation to the environment.

Clinical Perspectives summary

1. **Background:** Mesenchymal stem/stromal cells recruited in synovium (Synovium-Derived Stromal Cells-SDSCs), generally considered for their regenerative potential in cartilage lesions, could have a role in the onset/maintenance of OA. Telocytes (TCs) were also investigated.
2. **Results:** Our in vitro study showed that only the SDSCs harvested from OA subjects were capable to generate active osteoclasts from healthy donor PBMCs. TCs were more numerous in cultures obtained from OA in comparison to heathy subjects, suggesting their possible involvement in OA.
3. **Potential significance:** Cartilage regeneration strategies in OA must take into account the multifaceted capability of MSCs in relation to the environment.

1. Introduction

Osteochondral defects may progress in osteoarthritis (OA), which is one of the most common sources of articular pain and disability in an aging population [1]. To date, several therapeutic efforts have been made, but no treatment has been proven to stabilize, reverse or prevent OA development, and cartilage recovery continues to represent a challenge to scientists and clinicians [2]. OA is currently defined as a disease of the whole joint, as it affects not only the cartilage but also the subchondral bone and the synovial tissue [3]. Recent researches are focusing on an in-depth characterisation of cells harvested from the synovium (SM) of normal and OA subjects to elucidate their involvement in the regeneration and/or pathogenesis of joint diseases.

SM is a specialized mesenchymal tissue that includes two layers: the intima in contact with the articular cavity, which is composed of one or two cell sheets, and the underlying subintima, consisting of abundant collagenous extracellular matrix (ECM) with dispersed fibroblast-like cells, macrophages, mast cells, autonomic nerve fibers, and blood and lymphatic vessels [4,5]. The intima encompasses two morphologically different cell types named Type A and Type B synoviocytes. The former are bone marrow-derived phagocytic cells participating in the clearance of debris from the joint cavity and serving as immune sentinels. Type B synoviocytes, a kind of mesenchymal cells, are responsible for the production of synovial fluid molecular components, including hyaluronan and lubricin [4,5]. The subintima consists of a loosely organized and highly vascularized connective tissue that forms a support for the overlying intima [4–6]. It allows the transfer of molecular and cellular components from the circulation to the intima and synovial fluid in the articular cavity [7] and represents a potential reserve of type B synoviocytes for the maintenance of synovial intima integrity [7] . The subintima contains also immune competent cells.

Human SM hosts Mesenchymal Stromal Cells (MSCs) [8,9], called Synovial-Derived Stem Cells (SDSCs), that share the same phenotypic and functional properties of bone marrow (BM) MSCs [10]. Identification of MSCs in the synovium has raised speculations about their biological

involvement in the normal or pathologic joint physiology. Their possible role in synovial intima is related to their potential to differentiate into a wide variety of diarthrodial joint cell types (i.e. chondroblasts, osteoblasts and adipocytes). SDSCs were shown to have the greatest chondrogenesis potential among the mesenchymal tissue-derived cells, representing a possible source for cartilage repair [11]. SDSCs were also demonstrated to be superior in terms of adipogenesis [12] and, together with those of periosteum, were shown to be superior in osteogenesis [13,14]. These results indicate the therapeutic potential of SDSCs for the treatment of chondral defects. SDSCs can be found both in healthy and OA cartilage [11,13], and unlike BM MSCs, SDSCs seem to maintain an efficient proliferation rate and colony-forming potential regardless of the age of the patient [15]. Furthermore, these cells may be involved in early stages of osteartícular diseases [16]. Recently, the presence of a further stromal cell type with a peculiar ultrastructure called telocytes (TCs) has also been described in various human tissues including SM [17]. TCs possess very long and thin cellular extensions (telopodes) constituted by a repetition of thin segments (podomeres) and dilated portions (podoms) [18]. Their role is far to be clarified both in terms of tissue regeneration and disease occurrence.

Even if there are studies demonstrating the production of osteoclastogenic factors by synovial cells, not enough findings support the hypothesis that SDSCs could be implicated in OA pathophysiology. The purpose of our study was to isolate and culture SDSCs from healthy and OA SM and compare in vitro differences in term of morphology, phenotype, differentiation potential and capability to activate normal Peripheral Blood Mononuclear Cells (PBMCs).

2. Materials and Methods

2.1 Synovium (SM) and Synovial-Derived Stem Cells (SDSCs) isolation

SM was obtained during surgery for total knee arthroplasty in eight OA subjects (mean age 86±3), treated in the Azienda Ospedaliera-Universitaria Ospedali Riuniti of Ancona (Italy) from January 2015 to April 2017. Control SM was obtained from 2 healthy subjects, gender matching,

undergoing leg amputation. In accordance with the Local Ethical Committee guidelines and with the 1964 Helsinki declaration an informed consent was obtained from all individual participants included in the study. Patients were aware that the tissue used for the study represented a discard of surgical procedures and the voluntariness of their participation to the study (freedom from coercion or undue influence, real or imagined). SDSCs were isolated according to De Bari et al [8]: briefly, synovial tissues were rinsed with Dulbecco's Phosphate Buffered saline (DPBS) (Sigma-Aldrich, Milan, Italy), minced into small pieces and then digested with 0.2% collagenase (Collagenase NB 4G Proved Grade, Serva Electrophoresis GmbH, Germany) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Sigma-Aldrich), supplemented with 2% Foetal Bovine Serum (FBS) and 1% penicillin-streptomycin (100U/ml) at 37°C in a humified atmosphere, with 5% CO2. FBS and antibiotics were both from GIBCO® (Thermo Fisher Scientific, Waltham, MA, USA). After an overnight incubation, samples were filtered through 40μm nylon-mesh cell-strainers (BD Biosciences, San Jose, CA) to remove large debris. Single cell suspensions were cultured in DMEM/F-12 with 10% FBS and 1% antibiotics (from here on defined Complete Medium-CM) in tissue culture flasks, changing CM twice a week. Upon reaching 50% confluence, cells were carefully detached with 0.25% trypsin/1mM EDTA (Sigma-Aldrich). Non-adherent cells were gradually lost by medium changing. From each explant we were able to obtain an adequate number of cells without excessive subuculturing (i.e. within the 4th passage of subculture) allowing the set up two different sets of experiments.

2.2 SDSCs Characterization

Immunophenotype of synovial adherent cells was investigated by flow cytometry according to the minimal criteria of the International Society for Cellular Therapy (ISCT) [19]. The following fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies (all by Immunotools, Friesoythe, Germany) were used: anti-CD14 (Clone MEM-15), CD34 (Clone 4H11-APG), CD45 (Clone MEM-28), CD73 (AD2), CD90 (Clone 5E10, StemCell Technologies, Milan, Italy), CD105

(Clone MEM-226) and CD106 (Clone STA). As isotype controls, FITC-coupled nonspecific mouse IgG replaced the primary antibodies. For each sample, at least 10,000 events were acquired by FACSCalibur flow cytometry system (Becton Dickinson, CA, USA) and data were analysed using FCS Express 6 Plus Software (De Novo Software, CA, USA). Forward (FSC) monitored the cell volume and Side scatter (SSC) evaluated the internal complexity.

2.3 SDSCs in vitro differentiation

For in vitro chondrogenic, osteogenic and adipogenic differentiation, commercial kits from Life Technologies Corporation (Carlsbad, USA) were used according to manufacturer's instructions.

For in vitro, chondrogenic, osteogenic and adipogenic differentiation commercial kits from Life Technologies Corporation (Carlsbad, USA) were used according to manufacturer's instruction (for details see supplementary information). The chondrogenic potential of adherent cells isolated from synovia was assessed using a pellet culture system. In brief, 5 x 10^5 cells were centrifuged for 10 minutes (min) in 15 mL polypropylene tubes and then cultured for 14 days in 1mL of STEMPRO® chondrogenic medium (Chondrogenesis Kit, Life Technologies Corporation, USA), replacing medium every 3–4 days. Cell pellets were paraffin-embedded, cut into 3 μm thick sections, and then stained with Alcian Blue solution pH 1 (Bio-Optica, Milan, Italy). For immunohistochemistry, deparaffinized sections were incubated with mouse monoclonal antibodies against aggrecan (Clone 3H524, dil. 1:20, Santa-Cruz Biotechnology inc, Heidelberg, Germany) and type II collagen (Clone II-4C11, dil 1:10, Merck Millipore, Darmstadt, Germany). After overnight incubation at 4°C, the immune complex was evidenced by the streptavidin–biotin peroxidase technique (Envision peroxidase kit, DakoCytomation, Milan, Italy). After the incubation with 0.05% 3,3′-diaminobenzidine (Sigma-Aldrich) in 0.05M Tris buffer, pH 7.6 with 0.01% hydrogen peroxide, samples were counterstained with Mayer's haematoxylin (BioOptica) dehydrated in ethanol and coverslipped with Eukitt mounting medium (Electron Microscopy Sciences, PA, USA).

For osteogenesis, cells were seeded in chamber slides (NuncTM, Rochester, NY) at a density of 5 x 10^3 cells cm^{-2} in appropriate induction medium (STEMPRO® Osteogenesis Kit, Life Technologies). After 21 days, Von Kossa staining evaluated matrix mineralization.

Adipogenic differentiation was performed by culturing 2 x 10^4 cells cm^{-2} with STEMPRO® Adipogenesis Kit (Life Technologies) for 14 days and detected by Oil Red staining (Sigma-Aldrich) according to manufacturer's instruction. The appearance of cytoplasmic lipid droplets was also identified by immunohistochemistry using a mouse antibody against perilipin 1 (dil. 1:100; Abcam, Cambridge, UK) and evidenced as described above.

Cells maintained in CM represented the negative controls. Nikon DSVi1 digital camera and NIS Elements BR 3.22 imaging software (both from Nikon Instruments, Florence, Italy) were used for images acquisition.

2.4 Ultrastructural analysis

For Transmission Electron Microscopy (TEM), tissue and cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at 4°C, post-fixed in 1% Osmium tetroxide in 0.1 M cacodylate buffer for 30 min at Room temperature (RT), dehydrated in an acetone series (70%, 90% and 100%) and embedded in Epon resin (Fluka, Sigma-Aldrich). 100 nm ultra-thin sections were cut using a Diatome diamond knife (Diatome, Hatfield, PA, USA) on a Reichert-Jung ultramicrotome (Ultracut E, Reichert G, Wien, Austria). Sections were picked up on nickel grids and stained with alcoholic uranyl acetate and Reynold's lead citrate. Ultrastructural examination was performed using the Philips CM10 Transmission Microscope equipped with Megaview III digital camera (FEI Company, Eindhoven, The Netherlands).

For Scanning Electron Microscopy (SEM) cells were fixed as described above, dehydrated in increasing ethanol concentrations (25%, 50%, 70%, 80%, 95% and 100%), dried by evaporation of hexamethyldisilazane (HMDS), mounted on aluminium stubs, gold-sputtered and observed with a SEM Philips XL 20 (FEI, Milan Italy).

2.5 Osteoclastogenesis

In vitro osteoclastogenesis was induced in Peripheral Blood Mononuclear Cells (PBMCs) by SDSCs through a Transwell (Thermo Scientific™ Nunc™ Carrier Plate system, Milan, Italy) co-culture system. PBMCs were obtained from gender matching healthy donors using density gradient Ficoll/Paque method. In brief, peripheral blood was diluted 1:1 in DPBS, layered on Histopaque®-1077 (Sigma-Aldrich) and centrifuged at 400g for 30 min. PBMCs at the interface plasma/Ficoll were collected, washed in DPBS, suspended in α-MEM (Corning Inc., NY, USA) supplemented with 20% FBS, 1% antibiotics and 2 ng/mL of M-CSF (PeproTech EC, London, UK). Cells were then seeded at a density of $2x10^6$ cells/well on Aclar® Film 33C (EMS, Fort Washington, PA, USA) placed in a 12 wells tissue culture plates (TCPs). Cells were cultured for 6 days, removing non-adherent cells with media changes. SDSCs were then seeded in cell culture inserts (with pores 0.4 μm) of the 12 wells TCPs containing PBMCs, at a density of $2x10^5$cells/well (ratio 1:10). Co-cultures were maintained for 2 weeks. At each culture media refresh, adherent cells were observed by a light inverted microscope to assess multinucleated cell development. After 14 days, the inserts were removed, and cells in the wells analysed for cytoskeleton distribution and the IHC expression of Tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CTSK). For methodological details see Supplementary Information (SI1). TRAP- or CTSK- positive multinuclear cells that contained more than three nuclei were identified as osteoclasts and counted. Nikon DSVi1 digital camera and NIS Elements BR 3.22 imaging software were used for images acquisition.

For the immunostaining evaluation of TRAP and Cathepsin K four images at 20X magnification from each sample were analyzed in semi-quantitative manner. For each image, cells with more than 3 nuclei were examined for TRAP and CatK staining and ranked as: 0 (negative), 1 (weak staining), 2 (good staining), 3 (strong staining). The score was calculated considering the number of positive cells and the staining rank, and analysed by GraphPad PRISM 4 (https://www.graphpad.com/scientific-software/prism/).

188 The resorptive ability of generated osteoclasts was assessed putting dentine discs (1 cm in diameter
189 and 0.7 μm in thickness) at the bottom of the wells of the previously described co-culture systems
190 and observed with a SEM Philips XL 20.
191
192 **2.6 qRT-PCR**
193 The expression of genes involved in osteoclastogenesis (Interleukin 6 - IL-6, Receptor activator of
194 nuclear factor kappa-B ligand - RANKL and osteoprotegerin - OPG) was assessed in SDSCs before
195 and after co-culture with PBMCs. Total RNA was extracted from cell pellets using the PerfectPure
196 RNA cultured cell kit (5-Prime GmbH, Hamburg, Germany) according to the manufacturer's
197 instructions. RNA quantity and quality were evaluated by UV spectrophotometric analysis
198 (bioPhotometer plus, Eppendorf GmbH, Germany). Standard reverse transcription was performed
199 using the GoScript TM reverse transcription system (Promega Corporation, Italy) starting from
200 1.0μg of total RNA. Each real-time quantitative PCR assay was executed in triplicate in white
201 plastic ware using the Mastercycler Realplex2 (Eppendorf GmbH). A final volume of 10μl with
202 1μL of cDNA (corresponding to 50 ng of total RNA template), 1X SsoFastTM EvaGreen ®
203 Supermix (Bio-Rad), and 200nM primers were used for PCR. The cycling conditions included an
204 initial step at 95 °C for 30 s, followed by 40 cycles at 95°C for 5s and at 60°C for 20s.
205 Oligonucleotide sequences were designed with Primer 3 (v. 0.4.0) software: Interleukin (IL)-6
206 (Forward: CCAGAGCTGTGCAGATGAGT; Reverse: CATTTGTGGTTGGGTCAGGG),
207 Osteoprotegerin - OPG (Forward: TGATGGAAAGCTTACCGGGA; Reverse
208 CAGGATCTGGTCACTGGGTT); Receptor Activator of Nuclear Factor κB ligand – RANKL
209 (Forward: TAATGCCACCGACATCCCAT; Reverse; ATGTTGGAGATCTTGGCCCA). To avoid
210 sequence homologies to pseudogenes or other undesired targets, primer specificity was checked by
211 BLAST. Melt curve analysis confirmed PCR specificity. Reference genes and each gene of interest
212 were amplified simultaneously under the same conditions in each PCR assay. Primers showed the
213 same amplification efficiency. Glyceraldehyde 3-phosphate dehydrogenase – GAPDH (Forward:

AGCCACATCGCTCAGACAC; Reverse: GCCCAATACGACCAAATCC) and beta-glucuronidase – GUSB (Forward: AAACGATTGCAGGGTTTCAC; Reverse TCTCGTCGGTGACTGTTCA) were used to normalize cellular mRNA data [20]. Normalization involved the ratio of mRNA concentrations of genes of interest (Ct values) to that of reference gene Ct medium values. Data were expressed as relative gene expression ($2^{-\Delta Ct}$). Each assay was performed in triplicate. To point out the effect of the different origin (OA vs H) or the co-culture system on SDSCs, ΔΔCt method for Fold-Change evaluation was used [21]. The qPCR efficiency in all our experiments was more than 90%.

2.7 Statistical analysis

Statistical analysis was performed by Prisma 4 Software (https://www.graphpad.com/scientific-software/prism/). Mean and standard deviation of two different experiments for cells obtained from each subject were analyzed by Mann–Whitney U test. Statistical significance was tested at $p < 0.05$.

3. Results

3.1 Synovium

Semithin sections of OA and healthy SM were subjected to toluidine blue staining to examine their general morphological features, followed by ultrastructural analysis of ultrathin sections to investigate the presence TCs. Cells with long and thin cytoplasmic processes were observed in toluidine blue-stained synovial semithin sections.

TEM observation evidenced the presence of Type B synoviocytes, characterized by a large body rich in mitochondria and cisternae of rough endoplasmic reticulum (RER), a large Golgi apparatus and short and thick processes. Synovial TCs generally showed a relatively large and slightly indented euchromatic nucleus, with patches of heterochromatin near the nuclear membrane. The cytoplasm contained few mitochondria, few RER cisternae and a small Golgi apparatus. Telopodes,

with a narrow emergence from the cellular body, were also evident. They displayed a repetition of extremely slim segments (podomers) and expanded parts (podoms) with RER and mitochondria. In OA-SM, mastocytes were also evident (Figure 1).

3.2 SDSCs characterization and differentiation ability

No differences in the phenotypic expression of common MSC markers were detected between SDSCs isolated from Synovium of OA and healthy subjects. Cells were positive for CD73, CD90 and CD105 (>98% of positive cells), whilst they were negative for hemopoietic antigens CD14, CD34 and CD45 (positivity <2%) (Fig. 2a). On the contrary, significant differences between healthy and OA cells were detected for the expression of CD106 (42.75±2.14% vs 49.02±2.45%; $p<0.05$).

The analysis of cell size (FSC) and internal granularity (SCC) revealed that SDSCs obtained from OA subjects were significantly larger than the healthy counterpart (250.49±6.89 vs 216.98±12.77, $p<0.01$) and showed an increased internal complexity (385.32±10.77 vs 339.20±8.13, $p<0.01$).

No changes in the cell differentiation potential of healthy and OA SDSCs were detected (Fig 2b) as underlined by the comparable Alcian Blue staining and immunohistochemical expression of aggrecan, Von Kossa and Alizarin Red mineralization assays, and Oil Red O-staining and perilipin immunohistochemical expression (Fig. 2b).

3.3 SDSC ultrastructure

SEM observation showed the presence of cells with different morphologies. In healthy- derived cells both Synoviocytes A and B were detectable. Some SDSCs showed oligodendritic (2-3 extended cell processes) and polydendritic (more than 4 cell processes). This morphology was suggestive for TCs. TEM analysis corroborated SEM observation even if cell monolayer hampered the correct visualization of telopodes (Fig. 2c).

3.4 Osteoclastogenesis

No evident multinucleated cells were observed until day 8 of SDSCs/PBMC co-culture. After 3 days of co-culture, adherent cells in the lower wells consisted of a mixed population of fibroblast-like and round shaped cells. From day 8, several multinucleated cells appeared in both OA SDSCs and healthy co-culture systems (Fig. 3a). After 14 days, F-actin ring formation and cytoskeletal tubulin revealed the presence of plentiful F-actin ring positive cells containing more than 3 nuclei in both samples and zipper-like structures (Fig. 3a).

At 14 days multinucleated cells were positive for TRAP and CTSK in both OA co-culture and healthy system, with several $TRAP^{+}$ cells showing a slight albeit not significantly increase in OA SDSCs co-culture system. On the contrary, a decrease in the number of the CTSK+ was observed (Figure 3b). The presence of multinucleated cells and resorbing pits were confirmed by SEM observation of osteoclast-like cells induced by OA-SDSCs (Fig 3c).

3.5 qRT-PCR

The relative expressions of IL-6 (4-fold) and OPG (2-fold) were down-regulated in SDSCs harvested from OA-subjects in comparison with H-SDSCs (Table 1). On the contrary, RANKL mRNA evidenced a significant increase, which was further responsible for the increase of RANKL/OPG ratio in OA-SDSCs.

The mRNA expression of OPG and RANKL was evaluated in SDSCs also after co-culture (Table 1). The comparison with their expression before and after co-culture evidenced an up-regulation of RANKL mRNA expression in H-SDSCs (8-fold) and a slight albeit significant decrease in OA-SDSCs. A different behavior was detected for OPG mRNA that was up-regulated in both SDSCs after co-culture. These changes resulted in significantly higher RANKL/OPG ratio in H-SDSCs in comparison to OA cells (Fig.3).

4. Discussion

Osteoarthritis (OA) is the most common form of chronic joint disease, representing the leading cause of pain and disability in an aging population. Despite its association with the aging process [22], at present OA is recognized as a pathology of the whole joint, including closely related changes in cartilage, synovium and subchondral bone [23]. The synovium entails the synovial membrane, which encapsulates the joint providing structural support, synovial fluid for proper lubrication and nutrients essential for normal joint function [4]. The subchondral region consists of the cortical bone underlying the calcified cartilage (subchondral plate) and the subchondral trabecular bone. Whether cartilage damage in OA affects the underlying bone or vice versa is still a matter of debate. During disease progression, the structural changes in OA bone (e.g. increased number of trabeculae, altered mineralization or osteophytes) are an expected consequence of alterations in the cell-mediated bone remodeling process [24]. Therefore, the idea of osteoclasts being directly involved in OA cartilage degradation has gained increasing attention. It is well known that Peripheral Blood Mononuclear Cells (PBMCs) from OA subjects show a high osteoclastogenic differentiation capability [25]. Moreover, Type A synoviocytes isolated from pathological synovial membrane can differentiate into osteoclasts [26,27] and this process is stimulated by M-CSF produced by type B synoviocytes.

The other key cellular players in bone remodeling are mesenchymal stem/stromal cells (MSCs). The behavior of MSCs is linked to several factors, such as chronic inflammation and age, but the underlying mechanisms and possible roles of interaction between MSCs and other specialized cells remain undefined. MSCs exert their beneficial effects via secretion of bioactive molecules (paracrine action), which can be antiapoptotic, mitotic, supportive for tissue resident progenitors, angiogenic, immunomodulating, or chemoattractant. Moreover, they support osteoclastogenesis through producing the main osteoclastogenic cytokines, RANKL, as well as osteoprotegerin (OPG),

a soluble member of the tumor necrosis factor receptor superfamily that acts by disrupting the interaction between RANKL and RANK, thus inhibiting bone resorption. Therefore, MSCs could have a dual effect, by stimulating or inhibiting osteoclastogenesis, depending on the inflammatory milieu. MSCs are common residents of all joint tissues, including synovial tissue and fluid [28]. The exact origin of the latter is not well known, but SM is considered as the most likely one [29]. Conflicting results are reported regarding the isolation of functionally normal MSCs from patients with OA. For instance, Scharstuhl and coworkers [30] demonstrated that the chondrogenic potential of MSCs is independent of age or OA etiology, whilst the group of Murphy [31] displayed that cells harvested from patients with advanced OA showed reduced proliferative and chondrogenic activity. Moreover, select findings raise suspicion that systemic depletion and imbalance of MSCs may contribute to OA pathophysiology.

Since there is not yet enough evidence to support the idea that SDSCs are involved in OA pathophysiology, this phenomenon in our opinion merits further consideration. To this aim, in the present study, we compared SDSCs isolated from healthy and osteoarthritic subjects in terms of phenotype, morphology, gene expression and capability to induce osteoclastogenesis.

For the isolation of SDSCs, the same protocol developed by De Bari and coworkers was used [8]. Cytofluorimetric analysis for the expression pattern of the surface antigens [12] of healthy and pathological SDSCs was in line with that defined by previous studies [13]. Attention was paid to the comparison of the positivity for CD90 and CD105 between the two cell populations, considering the close association between these superficial markers and the chondrogenic potential of SDSCs [32,33]. Our results showed a high positivity for CD90 and CD105, like other studies, and the absence of significant differences in the percentages of positivity between healthy and OA-derived cells. Overall, these data suggest the remarkable and comparable chondrogenic differentiation capability of both of healthy and osteoarthritic SDSCs. This observation was corroborated by the *in vitro* chondrogenic differentiation test and strengthened the idea of the use of SDSCs for regenerative medicine approaches [34]. An interesting aspect was the increase in positivity for

CD106 in SDSCs derived from OA patients. The CD106 that is specific for MSCs allow us to ascertain the mesenchymal nature of the isolated cells and discriminate between type B synoviocytes and SDSCs. Since proinflammatory cytokines could enhance its expression level, it represents a good reflection of the endogenous inflammatory milieu to which the SDSCs derived from OA subjects are exposed [35]. Even if this marker has been associated with changes in osteogenic and adipogenic potentials [36,37] this was not proved by our data, which showed no differences between healthy and OA-derived cells.

From a morphological point of view, it is interesting to note that SDSCs of healthy and OA subjects share the same morphological typologies with roundish structures on the cell surface, which constitute specializations associated with exocytosis [38]. The peculiar oligodendritic morphologies observed in both healthy and OA cultures are suggestive for the presence of telocytes. These interstitial cells, characterized by long cytoplasmic processes named telopodes and recently demonstrated also in the synovial membrane [17], seem to be more numerous in culture obtained from OA subjects in comparison to the healthy ones. Since TCs play an important role in many pathological processes and in adaptive responses [39,40], their increased number in culture obtained from OA subjects suggests their possible involvement in OA.

Interesting results emerged from the analysis of genes involved in bone remodeling (i.e. RANKL and OPG). The comparison of their expression in cells of healthy and OA subjects showed a higher RANKL/OPG ratio in SDSCs of OA subjects. This occurrence, like what observed in periosteum-derived stem cells of elderly subjects [41], suggested a potential contribution of SDSCs in the activation of multinucleated cells (chondroclasts and/or osteoclasts), responsible for cartilage erosion or subchondral bone resorption during age-related joint diseases, such as OA. To the best of our knowledge, no studies evaluated the potential of SDSCs isolated from OA, to favor the differentiation of PBMCs in active osteoclasts. For this reason, we decided to deepen our knowledge by evaluating the capability of SDSCs to generate active osteoclasts, by co-culturing SDSCs harvested from healthy and OA synovial membranes with PBMCs from healthy donors. Our

trans-well approach showed the presence of paracrine signals that induced osteoclastogenesis. Immunofluorescence for cytoskeleton evidenced the presence of plentiful F-actin ring positive cells containing more than 3 nuclei in both samples and zipper-like structures [42], which were more numerous after the induction with OA-SDSCs. Resorption assays performed on dentin slices underlined that only paracrine signals derived from cells harvested by OA subjects were able to determine the formation of resorption pits. The immunohistochemical analysis for Cathepsin K and TRAP confirmed the differentiation of PBMCs into osteoclasts in both co-cultures and strengthened SEM observations. Both osteoclast populations express cathepsin K, with a higher positivity in cells stimulated by healthy SDSCs. Cathepsin K is a cysteine protease, markedly expressed in active osteoclasts, whose function is the degradation of collagen type I [43]. In the ruffled border, Cathepsin K can cleave the inactive form of TRAP to obtain the functionally active protein [44]. OCs derived from healthy SDSCs do not exhibit functional activity and, for this reason, they accumulate Cathepsin K in the cytoplasmic compartment. As concern TRAP, no significant differences in its expression were found between the two-different stimulations. It must be underlined that this enzyme is normally released in the ruffled border as an inactive monomeric form, but only the processing by Cathepsin K allow its activation and, consequently, active osteoclasts.

Conclusion

In conclusion, in this study, we ascertained that SDSCs stimulated osteoclastogenesis by means of soluble factors, but the osteoclast-like cells generated by healthy-SDSCs via trans-well assays, were dormant. OA-derived SDSCs have much greater potency in stimulating osteoclastogenesis than healthy-SDSCs. Based on our in vitro studies, it can be concluded that SDSCs have a dual effect on osteoclasts, and this effect is dependent on the microenvironment. It will be also of interest confirm our results with the recently developed “suspended synovium culture model” [45] to demonstrate the actual role of mobilized cells.

Overall, these observations may represent an interesting opportunity for the development of a holistic approach for OA treatment, that considers the multifaced capability of MSCs in relation to the specific environment. In this respect, further studies to investigating the possible role of telocytes in the onset/maintenance of the pathology as well as a new target for pharmacological approaches, are desirable.

Limitations of the study

Some limitation of our study should be noted. First, only a small sample number was studied. This was mainly related to the difficulties in sampling, mainly for healthy tissue. However, from each explant we were capable to obtain an adequate number of cells in order to set up two different sets of experiments. Moreover, no in vivo studies are included. Concerning the use of bone and joint diseases model mechanism (DMM), no described indirect co-culture approaches proposed as DMM for OA [46, 47] have been used, as they considered the different cell populations crosstalk. In this respect our study paves the way for the development of a new in vitro DMM for OA.

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Statement of author contributions:

Prof. Mattioli-Belmonte planned and oversaw the whole research; Prof. Gigante furnished tissue samples and clinical suggestion; Dr. Dicarlo executed cell cultures and flow cytometry study; Dr. Cerqueni performed immunohistochemical analysis; Prof. Teti was responsible for ultrastructural

investigation; Dr. Iezzi was responsible for qRT-PCR analysis; Prof. Falconi oversaw morphological analyses. All authors equally and competently contributed to the draft.

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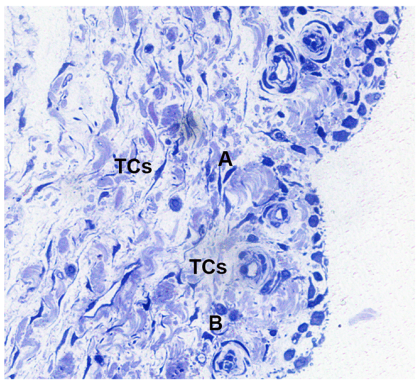
Figure legends:

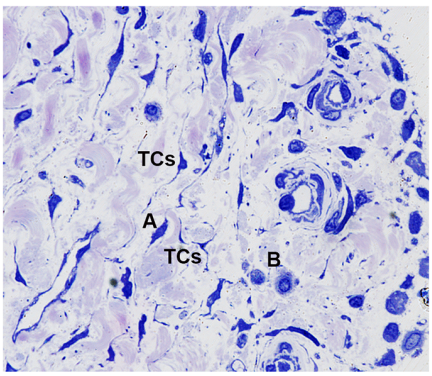
Figure 1: Representative semithin section of OA-SM. Types A and B synoviocytes as well as telocytes (TCs) are detectable. TEM morphological images of Types A and B synoviocytes, mastocytes and TCs present in OA -SM. N=Nucleus; G=Golgi apparatus; TP= telopodes; Pm=podomes; Pmr= podomers; Red arrows indicate lysosomes; Asterisks indicate rough endoplasmic reticulum.

Figure 2: A) Cytofluorimetric analysis of the detection of MSC surface markers in SDSCs isolated from Healthy and OA-SM (white plots indicate FITC negative controls), square brackets indicate significative ($p<0.05$) differences; B) Differentiation of SDSCs isolated from Healthy and OA-SM towards osteoblasts (Von Kossa and Alizarin red stainings; scale bars: 50μm), chondrocytes (Alcian blue staining and IHC for aggrecan; scale bars: 50μm) and adipocytes (Oil red staining and

IHC for perilipin; scale bars: 10µm); C) SEM and TEM representative images of cells isolated from Healthy and OA-SM; TCs=telocytes; Tp= telopodes.

Figure 3: A) Representative images displaying the generation of osteoclast-like cells after coculture of Healthy- or OA-SDSCs with normal PBMCs: Immunofluorescence for actin evidenced the appearance of the actin ring and of zipper-like structures (arrow); SEM observation on dentin slices were consistent with active osteoclast-like in OA-SDSCs/PBMCs co-culture; B) IHC detection of catepsin K (CTK) and TRAP in Osteoclast-like cells after 14 days of co- culture with Healthy- or OA-SDSCs, square brackets indicate significative ($p<0.05$) differences; C) Histograms depict changes in mRNA expression of genes involved in bone remodelling (Opg and RANKL) in Healthy- or OA-SDSCs, data are expressed as Fold-regulation ($2^{-\Delta\Delta Ct}$) (see Material and Methods); D) Histogram depicts changes in RANKL/OPG ratio in Healthy- or OA-SDSCs before and after co-culture. Scale bars: 50µm; square brackets indicate significative ($p<0.05$) differences.


TCs
A
TCs
B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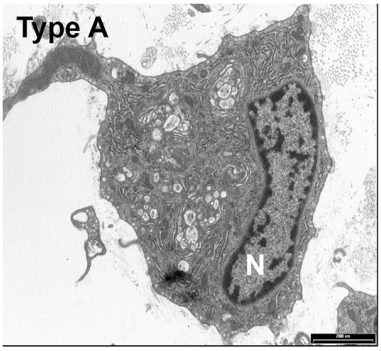
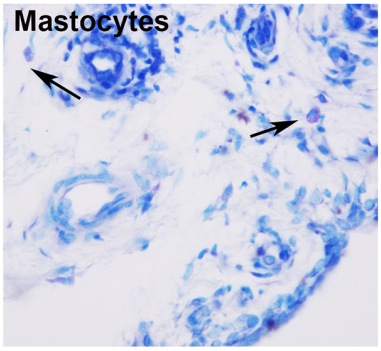
A
TCs
B


Type A
N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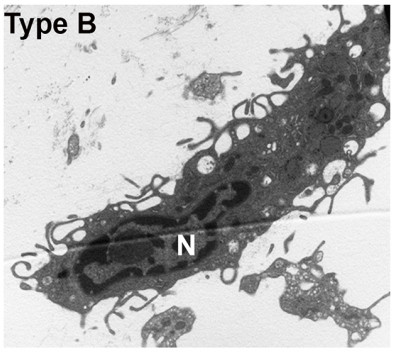
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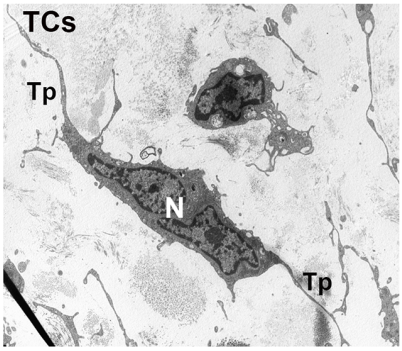
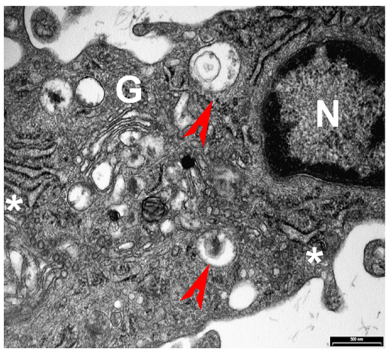

TCs
Tp
N
Tp


G
N
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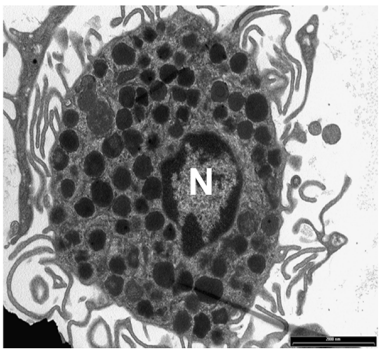
*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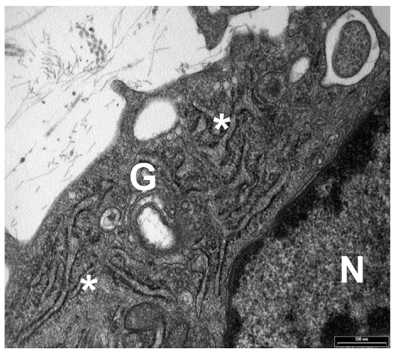
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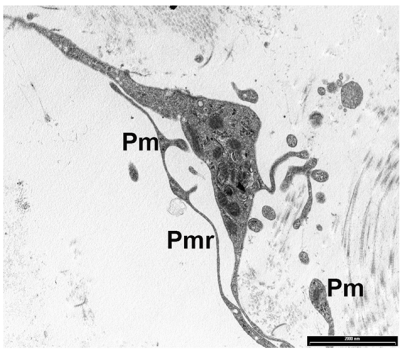
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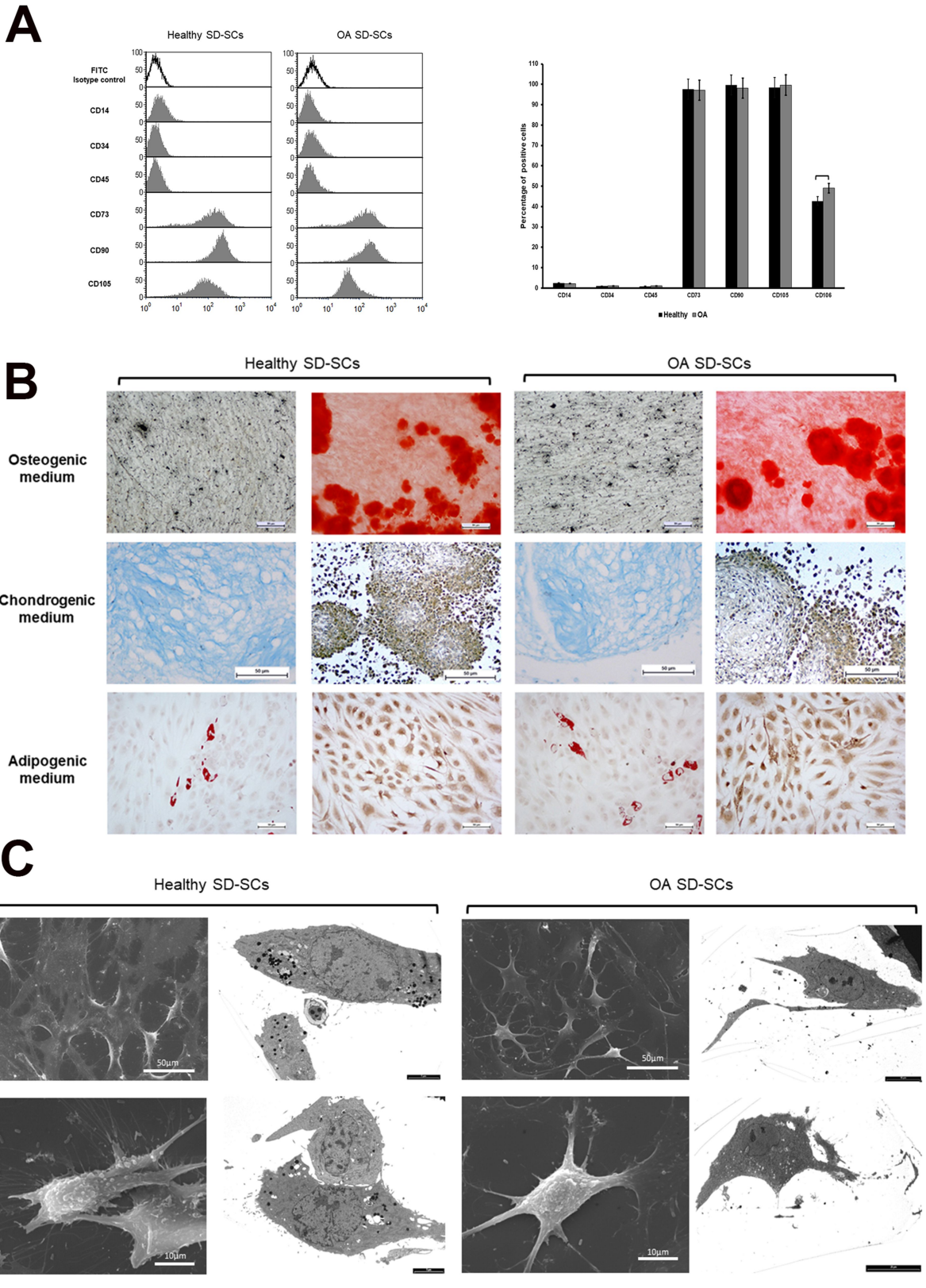
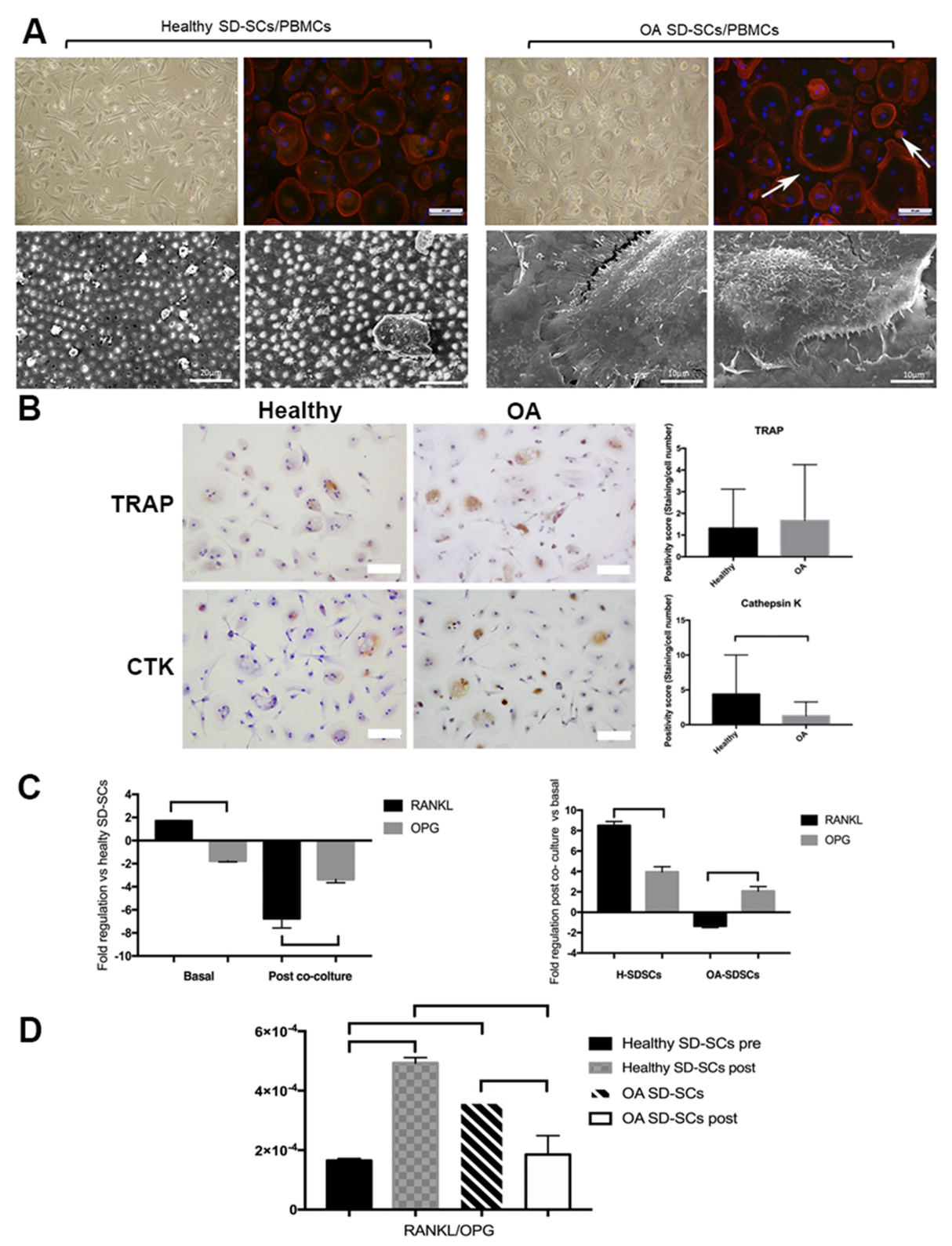
Pmr
Pm


A
Healthy SD-SCs
OA SD-SCs
FITC Isotype control
CD14
CD34
CD45
CD73
CD90
CD105
Percentage of positive cells
CD14
CD34
CD45
CD73
CD90
CD105
CD106
Healthy
OA
B
Healthy SD-SCs
OA SD-SCs
Osteogenic medium
Chondrogenic medium
Adipogenic medium
C
Healthy SD-SCs
OA SD-SCs
50μm
10μm


A
Healthy SD-SCs/PBMCs
OA SD-SCs/PBMCs
B
Healthy
OA
TRAP
CTK
TRAP
Positivity score (Staining/cell number)
Healthy
OA
Cathepsin K
Positivity score (Staining/cell number)
Healthy
OA
C
Fold regulation vs healty SD-SCs
RANKL
OPG
Basal
Post co-colture
Fold regulation post co- culture vs basal
RANKL
OPG
H-SDSCs
OA-SDSCs
D
Healthy SD-SCs pre
Healthy SD-SCs post
OA SD-SCs
OA SD-SCs post
RANKL/OPG